Quality Control of a Wheat Gluten Hydrolysate for Use as a Raw Material in Cell Culture Media

Heather Schwartz, Kevin Ray, Matt Caple, James Blasberg, and Benjamin Cutak
Sigma-Aldrich Corporation, St. Louis, Missouri

Abstract

A decrease in antibody productivity, with no corresponding decrease in cell growth, is associated with lot to lot variability of this hydrolysate. A reversed-phase HPLC method with photodiode array and MS detection has been developed for peptide mapping of wheat gluten hydrolysates in order to examine lot to lot variability. Significant differences noted in the profile have been correlated to observed differences in the biological activity of cells grown in medium containing different lots of hydrolysate. The presentation will demonstrate an LC-PDA-MS method that can be used as a quality control method for wheat gluten hydrolysates.

Introduction

Efforts to replace costly, undefined serum products in cell culture media have led to the study of low-cost protein hydrolysates from animal tissues, milk products, microorganisms and plant tissues.[1] Use of animal origin serum-replacement products has been re-evaluated due to increased concern about the potential for contamination from adventitious agents. Franek et al. reported that protein hydrolysates could either partially or fully replace serum as a concentrated balanced nutrient mixture.[2] The cell culture media developed by Sigma-Aldrich are frequently used in large-scale protein production by the biotechnology industry, which is increasingly requiring serum-free media. In an effort to replace serum in cell culture media, we have evaluated wheat gluten hydrolysates.

Wheat gluten hydrolysates are a known source of high levels of small peptides, larger oligopeptides, and are particularly rich in stable glutamine.[3] Sigma-Aldrich has observed the positive effect of wheat gluten hydrolysates on cell culture productivity, but the degree of productivity enhancement varies between lots of wheat gluten hydrolysate.

In general, the chemical composition of protein hydrolysates is somewhat ill defined. In order to control the quality of cell culture media formulated with protein hydrolysates, one must be able to control the quality of the hydrolysates. We report here the use of an LC/MS method with precise ion chromatograms to qualify wheat gluten hydrolysates as potential raw materials in serum-free cell culture media.

Materials and Methods

Chemicals and Equipment:
Sigma-Aldrich Corporation supplied all chemicals used, except the wheat gluten hydrolysate, which was obtained from Quest International. The HPLC system consisted of a Waters 2690 Alliance separation module equipped with a column heater coupled to a Waters 996-photodiode-array detector and a Micromass LCT mass spectrometer. A Supelcosil C18 column (25 cm x 4.6 mm; 5 µm) was used for the separation of the hydrolysates. Data collection and processing were performed using Waters Millennium software 3.05 and MassLynx 3.5.

Primary Chromatography (TFA Separation Method):
Mobile phase A was 0.1% trifluoroacetic acid (TFA) in HPLC grade water. Mobile phase B was 0.1% TFA in acetonitrile. Separation of hydrolysate components was performed using a 90 minute linear gradient from 0 to 30% B. The flow rate was 0.7 mL per minute and the column temperature was maintained at 25 °C. UV data were recorded from 210 nm to 285 nm. MS data were collected in positive electrospray mode using a capillary voltage of +3kV and cone voltages of 15-V and 30-V and recorded from m/z 100 to 1500. Leucine-enkephalin ([M+Na]$^+$ = 578.2590) was infused post-column as the lock mass for accurate mass measurements. The injection volume was 100 µL.

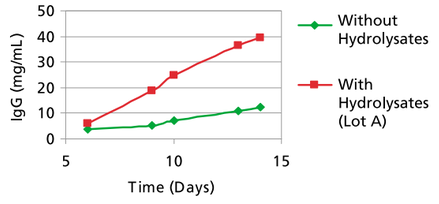
Secondary Chromatography (Phosphate Separation Method):
Mobile phase A was 25 mM Sodium phosphate buffer, pH 6. Mobile phase B was acetonitrile. Separation of hydrolysate components was performed using a 21 minute linear gradient from 0 to 7% B. The flow rate was 0.7 mL per minute and the column temperature was maintained at 25 °C. The injection volume was 100 µL. The UV and MS data were obtained as described above.

Cell culture:
A Chinese Hamster Ovary (CHO) cell line expressing a proprietary recombinant antibody was cultured in CHO Protein-Free Animal Component-Free Medium (Sigma C5467) supplemented with 2.5 g/L of wheat gluten hydrolysate. The cultures, in 100 mL Techne spinner flasks, were kept at 37 °C in a humidified atmosphere containing 5% CO_2. Quantitation of the recombinant antibody was performed by affinity HPLC using a Protein G column from Applied Biosystems.

Results and Discussion

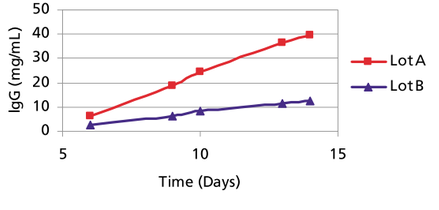
Wheat gluten hydrolysate is an enzymatic digest of wheat gluten that provides a high quality source of peptides, which is particularly rich in stable glutamine.[3] During the course of serum-free medium development for CHO cells, the incorporation of this wheat gluten hydrolysate in cell culture media was found to have a positive effect on protein expression versus no supplementation with the hydrolysate (Figure 1A). This effect was reproduced in many other CHO cell culture experiments in which supplementation with 2.5 g/L wheat gluten hydrolysate was found to be optimal. Several lots of this hydrolysate were analyzed in experiments designed to further demonstrate a correlation between cell culture productivity and the presence of this hydrolysate. These experiments showed that different lots (Lot A and Lot B) of wheat gluten hydrolysate produced different degrees of antibody production (Figure 1B).

Figure 1A: Effect of the Presence of Wheat Gluten Hydrolysates on Antibody Production



Several lots of this hydrolysate were analyzed in experiments designed to further demonstrate a correlation between cell culture productivity and the presence of this hydrolysate. These experiments showed that different lots of wheat gluten hydrolysate produced similar cell densities, but different degrees of antibody production were observed (Figure 1B).

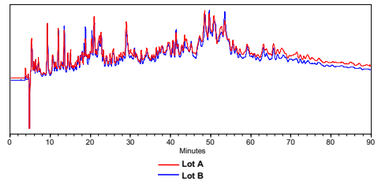
Figure 1B: Variation in Antibody Production with Respect to Lot of Wheat Gluten Hydrolysate



Because the raw material was a protein hydrolysate, the investigation began with amino acid composition analysis. The amino acid composition analysis of two lots of wheat gluten hydrolysate that resulted in different cell culture productivity enhancements showed no significant differences. Similarly, no significant differences were observed in the elemental composition of the two lots obtained by ICP atomic emission spectroscopy.[4] This data seemed to indicate that both lots of hydrolysate were produced from similar wheat gluten preparations and that the productivity differences observed for the wheat glutens were likely related to the peptide composition arising from the duration of hydrolysis, temperature applied during hydrolysis, or pH of the hydrolysis reaction.

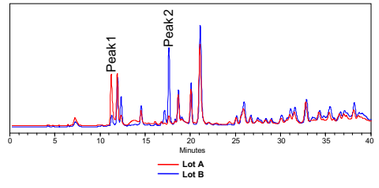
The TFA separation method was developed to qualitatively evaluate differences in the peptide composition of wheat gluten hydrolysates. The method employed a shallow gradient because of the high concentration of small hydrophilic peptides in the hydrolysates. Initially the absorbance at 214 nm was monitored because it is generally accepted as the standard peptide-mapping wavelength.[5] Figure 2A shows the TFA chromatograms at 214 nm obtained for two lots of wheat gluten hydrolysate that resulted in different cell culture productivity enhancements. It is apparent that both hydrolysates contain a considerable number of distinct peptide components. The nearly identical overlay of the two lots of hydrolysate at 214 nm does not provide any significant differentiation between the samples (Figure 2A).

Figure 2A: Overlaid TFA Chromatograms (214 nm) for Two Lots of Wheat Gluten Hydrolysate



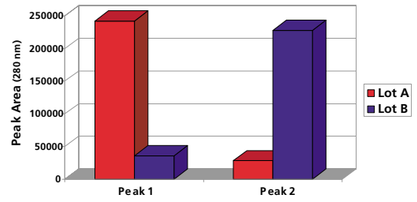
Because the spectral range from 210 nm to 285 nm was collected throughout the separation using a photodiode array detector, chromatograms at wavelengths other than 214 nm could be extracted and compared in search of differences between the hydrolysate samples. The UV spectra of proteins and peptides are greatly dependent on the spectra of the amino acids of which they are composed.[6] The absorbance at 280 nm is often used for protein and peptide detection based on the aromatic amino acid residues. Figure 2B shows a portion of the 280 nm chromatograms extracted from the same data set as Figure 2A. The two peaks demonstrating the greatest difference between the samples are arbitrarily labeled as Peak 1 and Peak 2 as a designation for further studies.

Figure 2B: Overlaid TFA Chromatograms (280 nm) for Two Lots of Wheat Gluten Hydrolysate



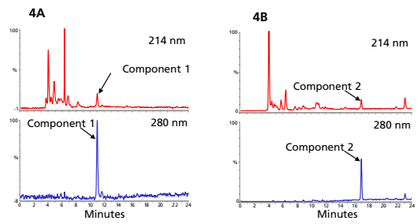
Comparison of the areas of Peak 1 and Peak 2, as illustrated in Figure 3, shows a distinct difference between the two lots. The area of Peak 1 seems to provide a parameter that is correlated to the biological activity of the hydrolysate; however, the area of Peak 2 appears to be inversely related to biological activity (refer to Figure 1B and Figure 3). In short, more Component 1 and less Component 2 seems to result in the greatest productivity.

Figure 3: Comparison of Peak Areas Between Two Lots of Wheat Gluten Hydrolysate Using TFA Separation Method



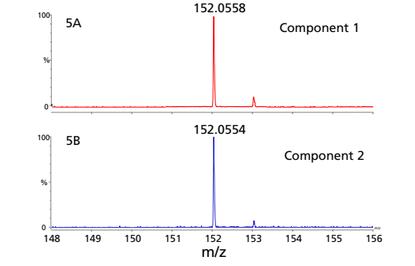
Attempts to assign a mass to Peak 1 and Peak 2 by LC/MS using the TFA separation method were inconclusive due to the complexity of the mass spectra resulting from the coelution of the components of interest with numerous, more abundant non-aromatic peptides. Fractions corresponding to Peak 1 and Peak 2 were collected from the TFA separation method. The TFA fractions were then separated using a phosphate-buffered mobile phase at pH 6. Inspection of the 214 nm chromatograms reveals that the TFA fractions were indeed comprised of a number of components that can be resolved by the phosphate separation method (Figures 4A and 4B). The 280 nm chromatograms of the TFA fractions show single chromatographic peaks corresponding to the components of interest (Figures 4A and 4B).

Figures 4A and 4B: Phosphate Chromatograms of Peaks 1 and 2 at 214 and 280 nm



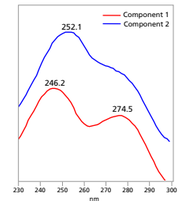
Since the components of interest were now isolated from the non-aromatic components in the TFA fractions, we were able to obtain photodiode array (PDA) spectra and accurate mass spectra of Components 1 and 2 using the phosphate separation. The chromatographic peaks for Components 1 and 2 demonstrate good peak purity by PDA and MS analysis. The exact mass of the peak at 11.07 minutes in Figure 4A was determined to be m/z 152.0558 (Figure 5A). The exact mass of the peak at 17.00 minutes in Figure 4B was determined to be m/z 152.0554 (Figure 5B).

Figures 5A and 5B: Exact Mass Spectra of Component 1 and Component 2 Using Phosphate Separation Method



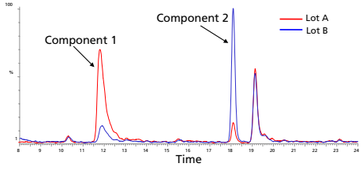
The mass assignments for Component 1 and Component 2 are within the mass accuracy specifications of the LCT instrument (± 2 mDa) below m/z 400. Despite the equivalent mass assignments, it is interesting to note that the PDA spectra of Components 1 and 2 are distinct (Figure 6)

Figure 6: PDA Spectra of Components 1 and 2



Using the mass assignments established using the phosphate separation, it was possible to extract ion chromatograms for Components 1 and 2 in wheat gluten samples separated using the TFA separation alone. Ion chromatograms reconstructed using a narrow mass range (± 0.05 Da) demonstrate improved selectivity and signal-to-noise ratios for Component 1 and Component 2 when compared to ion chromatograms reconstructed using nominal mass assignments. Figure 7 shows the chromatograms extracted at m/z 152.05 ± 0.05 of the same wheat gluten hydrolysates shown in Figures 2A and 2B. The peak area ratio of Components 1 and 2 in the extracted ion chromatograms in Figure 7 is equivalent to the ratio of Peaks 1 and 2 shown in Figure 3. The extracted ion chromatogram analysis is preferred because it is more selective than the 280 nm analysis and more robust than the 280 nm analysis due to the lesser demands on chromatographic resolution. While we have not established the absolute identity of Component 1 or Component 2, we have developed a robust LC/MS quality control procedure that can be used for the profiling and release of wheat gluten hydrolysates for use in cell culture medium production.

Figure 7: Overlaid TFA Extracted Ion Chromatograms at m/z 152.05 for Two Lots of Wheat Gluten Hydrolysate



References

(1) Schlaeger, E. The Protein Hydrolysate, Primatone RL, is a Cost-Effective Multiple Growth Promoter of Mammalian Cell Culture Serum-Containing and Serum-Free Media and Displays Anti-Apoptotic Properties, *J. Immunol. Methods* **1996,** *194,* 191-199.

(2) Franek, F., Hohenwarter, O. and Katinger, H. Plant Protein Hydrolysates: Preparation of Defined Peptide Fractions Promoting Growth and Production in Animal Cells Cultures, *Biotechnol.* **2000,** *16,* 688-692.

(3) Blom, W., Kunst, A., van Schie, B J. and Luly, G.W. US Patent 5,741,705 **1998.**

(4) Schwartz, H., Ray, K. and Caple, M. Peptide Mapping Of A Wheat Gluten Hydrolysate By Reversed-Phase High Performance Liquid Chromatography (RP-HPLC), Poster Presentation Number 202 at the 36th Midwest Regional Meeting of the American Chemical Society.

(5) Young, P.M. and Burgoyne, R.F. Photodiode array spectral identification of modified amino acid synthetic peptides following high resolution HPLC separation, *Pept. Res.* **1990,** *3,* 81-84.

(6) Zavitsanos, P. and Goetz, H. (1991) The Practical Application of Diode Array UV-Visible Detectors to High-Performance Liquid Chromatography Analysis of Peptides and Proteins. *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation.* In C.T. Mant; R.S. Hodges (Eds.) pp. 553-562.